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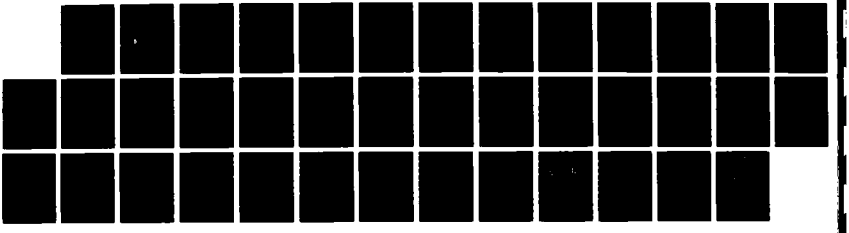

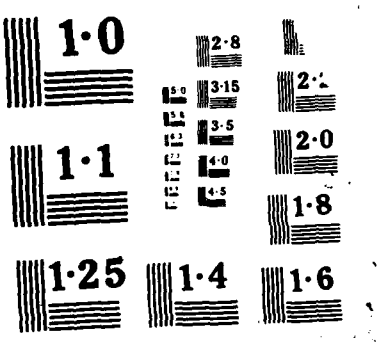
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Combination Chemotherapy Using Immune Modulators and Antiviral Drugs Against Togaviruses and Bunyaviruses

Subtitle: Prescreening and Test Systems

ANNUAL REPORT

D.H. Coppenhaver, M. Sarzotti, I.P. Singh, H. Lucia and S. Baron

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Infections caused by exotic viruses are an important publich health problem worldwide, and are a threat to become more widely desiminated. Representative viruses from four medically important groups were chosen as model systems to investigate potential antiviral therapies. Select Alphaviruses (Semliki Forest, Sindbis), Flaviviruses (Banzi, West Nile), Bunyaviruses (Bunyamwera, LaCrosse), and an Arenavirus (Pichinde) were studied. Model systems were established in vitro and in vivo. In vitro screens of candidate antiviral substances identified compounds of potential therapeutic interest. The most promising compounds were examined in vivo, using Semliki Forest and Banzi weanling mouse models, to establish minimal therapeutic levels. These experiments will form the basis for designing combination therapies using immune modulators and antiviral drugs.

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SUMMARY

Model systems for the *in vitro* and *in vivo* study of Alphaviruses (Semliki Forest, Sindbis), Flaviviruses (Banzi, West Nile), Bunyaviruses (Bunyamwera, La Crosse) and Arenaviruses (Pichinde) were established. Candidate antiviral compounds were screened using three tissue culture assay systems: pretreatment assay (interferon type), brief exposure cocultivation assay (2 hr decanting assay), and continuous cocultivation assay. On the basis of *in vitro* results, the dose responses of candidate compounds were determined *in vivo* using weanline mouse model systems. Data from these ongoing experiments are used to design experiments testing the interactions of individual antiviral drugs and immune modulators when used in combination.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or service of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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Introduction and Rationale

The research being pursued under this contract has the ultimate goal of designing and identifying effective therapeutic and prophylactic regimes for the treatment of medically and militarily important viral infections. Specifically, we are concerned with Alphaviruses, Flaviviruses, Bunyaviruses, and Arenaviruses. It was the primary goal of the initial contract year to establish test systems in vitro and in vivo to allow us to investigate antiviral agents against representatives of each of these groups. The test systems would then be used to screen a variety of candidate antiviral drugs for efficacy in vitro. Promising substances would then be evaluated in primary test systems in vivo. Results from in vitro and in vivo efficacy, toxicity, and dose response experiments can then be used to design experiments to test the relative effectiveness of combinations of antiviral drugs and immunomodulators in the treatment of viral infections.

Experimental Methods.

Viruses. Seed Alphaviruses [Semliki Forest (SF), Sindbis (SB)], Flaviviruses [Banzi (BZ), West Nile (WN)] and Bunyaviruses [Bunyamwera (BW), LaCrosse (LAC)] were received from Dr. Robert Shope of the Yale Arbovirus Unit. Viruses were passaged in newborn mice to prepare virus stocks. Briefly, viruses were inoculated intracranially into suckling mice 1-2 days postpartum. At the first sign of illness, generally 2-4 days after virus challenge, the mice were sacrificed and a 10% brain suspension in Eagles Minimum Essential Media was prepared. Some mice were frozen (-70°C) immediately after sacrifice, for use in antibody preparation. The 10% brain suspensions were aliquoted and frozen (-70°C) for future use.

Pichinde virus was received from Dr. David Gangemi, University of South Carolina. The virus (0.1 ml) was inoculated i.p. into 450-500 g Strain 13 guinea pigs. The animals were sacrificed on day 6 post infection, and 10% spleen suspensions prepared. This virus suspension was repassaged in Strain 13 guinea pigs to prepare the final stock virus (passage 14), which was aliquoted and frozen (-70°C).

Viral Pathogenicity. Dilutions of stock arboviruses were inoculated into outbred, weanling mice (Strain ICR) (15-22 g) intraperitoneally (i.p.) for LD_{75} determinations. SF and BZ viruses produced lethal encephalitis at days 5-7 and 6-9, respectively, with 0.2 ml of a virus dilution of $10^{-6.5}$ producing 1 LD_{75} in each case. SB, WN, BW and LAC gave irregular results when injected i.p., but produced the expected pathogenesis when given intracranially. Suckling mice are currently being evaluated for susceptibility to these viruses after i.p. challenge.

Pichinde virus (PIC) dilutions were inoculated i.p. into groups of strain 13 guinea pigs weighing 450-500 g. All animals died between days 13-19 after virus challenge, except for one in the 10^{-4} dilution group. Thus, the LD_{50} of our stock PIC appears to be less than 0.1 ml of a 10^{-4} dilution.

Tissue Culture Systems. Tissue culture systems for the test viruses are outlined in Table 1. In general, three different in vitro assay systems are used to evaluate the relative antiviral effects of the candidate compounds. The first, interferon-type assay involves preincubation (24 hr) of the host cell

monolayer with the compound of interest. The drug is then removed from the culture, and the monolayers challenged with virus. Viral plaque formation is evaluated after a further 24-48 hr incubation. In the second assay system cells are co-incubated with the drug dilutions and challenge virus for two hours. The monolayers are then decanted, removing unbound virus and the test drug, followed by addition of a methyl cellulose solution in media. The methyl cellulose overlay is added to increase the viscosity of the media, thereby decreasing virus spread and allowing the formation of discreet, countable virus plaques. During the remainder of the 24-48 hr incubation, the drug is removed from the system. The third assay system is analogous to the second (decanting) system, in that cells are not pretreated with the antiviral compound. In this continuous overlay system, the drug is not decanted from the monolayer after a 2 hr incubation. Instead, a more concentrated methyl cellulose overlay is used to promote plaque development.

Antibody Production. Immune globulins were prepared by hyperimmunization of ICR (outbred) mice. Virus stocks (10% brain suspensions) were inactivated by incubation with β-propriolactone (37°, 2 hr), and diluted with Freund's complete adjuvant (1:1). The mixture (0.5 ml) was injected i.p. into adult mice. Antigen boosts were given at days 7, 14, 21 and 28. Ascitic fluid from immunized mice was withdrawn ad libitum, and antigen boosts were given as needed to stimulate production of ascitic fluid.

Animal Models. Protection against arbovirus infections is evaluated in vivo using weanling mouse models. Outbred mice, strain ICR, at 18-22 g (4-5 weeks) are inoculated with the drug to be evaluated. Route and schedule of drug delivery are dependent on the specific compound being evaluated. Virus challenge (1-3 LD_{75}) is administered i.p. Primary evaluation is by presence of an amelioration of lethal encephalitis. Mice are generally maintained for 12-14 days after virus challenge before an experiment is terminated, but all groups are held for at least 3 days after the last recorded death. Pichinde virus is administered i.p. to 450-500 g strain 13 guinea pigs, as outlined above.

Results

Antiviral Assays. Four interferon (IFN) preparations were assayed for inhibition of arbovirus replication in vitro: natural murine IFNα/β, natural murine IFNγ, human recombinant IFNα A/D, and natural human IFNγ (Table 2). The observed level of the antiviral effect of these interferons is clearly influenced both by the degree of susceptibility of the target virus to the protective effects of interferon and by the (often limited) amount of cross species activity shown by the interferons. The latter is dependent both on the origin of the interferon and the cell culture system used in the assay.

Non-interferon antiviral compounds were screened using both the 2 hr decanting and the continuous overlay assays. Results for the 2 hr decanting assay are shown in Tables 3 and 4, while results for the continuous overlay system are given in Tables 5 and 6. Some compounds did not show any antiviral effects beyond their toxic levels on particular cell lines. These drugs are indicated with minimum inhibitory concentrations (MIC) of greater than the toxic concentration (Tables 3,5); or with virus plaque reduction titers of less than the toxic level (Tables 4,6).

Antibody Production. Antibodies were produced for all six arboviruses to be used in this research. High titer antibody pools were produced for all except the two alphaviruses, both of which induce low levels of neutralizing antibody, as has also been observed elsewhere (Table 7). Pichinde virus stock was treated as described above. The inactivated virus was mixed with Freund's complete adjuvant (1:1); 1 ml was injected i.p. weekly, for two weeks, into Hartley guinea pigs (500 g). All animals died of progressive viral disease within 12-16 days of the initial inoculation. Further attempts at antibody production are being pursued.

Viral Pathogenicity. Virus stocks of BZ and SF viruses produce lethal encephalitis 6-9 days after injection i.p. into weanling ICR mice. Stock WN, LAC, and BW viruses (0.03 ml) produce lethal encephalitis within 3-5 days when injected intracranially into 3-4 week old (16-17 g) ICR mice. Experiments to determine correct dosages and age of target animals for i.p. challenge with these viruses are ongoing. Histologic evaluation of arbovirus infections are also ongoing.

Twelve strain 13 guinea pigs (450-500 g) received 0.1 ml stock virus (passage 14) i.p. Animals were weighed three times a week. On days 4, 7, 11 and 15, animals were sacrificed, the blood collected in citrated collection tubes for clotting studies, and the organs processed for light and electron microscopy. The animals all lost a marked amount of weight (Figure 1). Their bodies seemed to lose muscle mass as well as fat. One animal died on day 13, and the remainder were either dead (3) or moribund (2) on day 14 post infection. The clotting studies revealed marked prolongation of the prothrombin (PT) and partial thromboplastin (APPT) times by Day 7 post infection (Figure 2). These abnormalities continued until death. Further evaluation of individual clotting factors is currently underway. However, the abnormalities seemed extreme for the amount of pathology seen in the liver and other tissues.

Lymph nodes, spleen, lung, liver, kidney, pancreas, and adrenal tissue were examined after fixation in neutral buffered formalin and routine processing for light microscopy. On Day 4, in the liver, a few foci of single-cell hepatocyte necrosis, surrounded by fragmenting PMNs were noted. All other organs were unremarkable. On Day 7, in the liver, these foci were more numerous. Cells were noted in which the nuclei had lost all stainable chromatin. These hollow nuclei resemble cells infected with HSV. The spleen and lymph nodes had decreased numbers of mature lymphocytes. A few areas of eosinophilic necrosis, with "hollow nuclei" at the margins were noted in the lymph nodes.

On Day 11, the animals had interstitial pneumonia, with both PMNs and macrophage infiltration of the septae. The livers showed moderate fatty metamorphosis, in addition to foci of necrosis with PMN infiltration. The kidney tubular epithelium showed foci of single-cell necrosis, especially in the straight tubules. The spleen and lymph nodes showed further loss of mature lymphocytes, with areas of necrosis in the periarteriolar lymphoid sheaths, encircling the lymphoid follicules. The adrenals had scattered foci of necrosis in the cortex with PMNs encircling the debris. All areas of necrosis were bordered by cells with "hollow nuclei".

On Day 14, the animals had moderately severe interstitial pneumonia. The livers had severe fatty metamorphosis, and many patches of necrosis. Fibrin deposition was noted in the areas of necrosis. The lymph nodes and spleens had

marked loss of mature lymphocytes, and large numbers of PMNs and nuclear debris in patches of necrosis. The pancreas of two animals showed necrosis of the islet cells. The adrenals had many areas of necrosis with PMN infiltration.

It was notable that there were no areas of mononuclear cell infiltration in these organs. This is distinctly unusual for viral infections. The PMNs appear to be there in response to tissue necrosis, but there is no cellular reaction to the viral antigen that must be present in large quantities. NK function seems suppressed.

Single Drug Dose Response Experiments In Vivo. Single drug protection experiments using the SF model system were run using 6 candidate antivirals: NH_4Cl, chloroquine, murine IFNα/β, non-peptide CVI, Ampligen and CL-246,738. Partial protection was seen for NH_4Cl and non-peptide CVI. Strong protection was seen for Ampligen (Table 8), murine IFNα/β (Table 9) and CL-246,738 (Table 10), although a complete end point (non-protective dose) was not obtained for CL-246,738. Similar experiments using the BZ model system were done using chloroquine, immune globulin, murine IFNα/β, Ampligen, poly I:CLC, and CL-246,738. Partial protection was seen with chloroquine (Table 11); no protection was seen for murine IFNα/β (Table 12); and strong protection was noted for antibody (Table 13); Ampligen (Table 14), poly I:CLC (Table 14), and CL-246,738 (Table 15). All of these results are being confirmed and, where complete dose response data have not yet been obtained, extended.

Combination Chemotherapy In Vivo. Based on our preliminary single drug titrations, we have begun combination experiments in vivo. Initial experiments are designed as a 4 x 4 grid, using three doses of each test compound, as well as single drug controls. In addition, a drug toxicity control is included in all initial combinations. The protocol is outlined in Table 16, which shows results of combining anti-Banzi immune globulin with poly I:CLC. This experiment gave some indication of greater than additive protection, but will need to be repeated with lower levels of the two antiviral agents in order to confirm this result.

Interferon Induction by CL-246,738. Orally administered CL-246,738 was effective in protecting weanling mice from lethal SF- and BZ-produced encephalitis (Tables 10,15). Since the drug was suspected of being a potent inducer of IFN, we investigated IFN levels in mice given CL-246,738. Following oral administration of CL-246,738, the serum IFN titer reached a peak 24 hrs after treatment and declined thereafter (Fig. 3). Challenge of the treated mice with a second dose of the drug, 24 hrs or 48 hrs after the first dose, did not boost the serum IFN response. High levels of IFN were also found in peritoneal lavage fluid of mice treated orally 24 hrs earlier, with CL-246,738 (Fig. 4). IFN was never detected in the serum or in the peritoneal fluid of mice which had been injected with the drug 6 hrs earlier.

We then investigated which cells were responsible for the production of IFN in vivo. Spleen or peritoneal exudate cells (PEC), obtained from mice injected 6 or 24 hrs earlier with the drug, were cultured in vitro for 24 hrs. Culture supernatants were then tested for IFN activity. As shown in Figure 5, low levels of IFN were detectable in the culture supernatants of spleen cells obtained from mice 6 hrs after treatment with CL-246,738. Titers from spleen cell supernatants were maximal when cells were harvested 6 hrs after treatment of the mice. In contrast, high levels of IFN were produced when PEC were

harvested from mice 24 hrs after CL-246,738 treatment. As observed in the serum, no detectable IFN was produced by PEC 6 hrs after the drug administration (Fig. 5). Interestingly, the highest IFN response was obtained with PEC from mice treated with the lowest dose of CL-246,738 (0.1 mg/mouse).

CL-246,738 was also tested in tissue culture systems in order to design drugs combination experiments in vivo. We studied whether murine or human lymphocytes could be induced in vitro with CL-246,738 to produce IFN (Table 17). In contrast to the results reported for the induction in vivo with CL-246,738, when murine cells were treated in vitro with CL-246,738, inconsistent results were obtained. Some IFN was obtained from PEC in exp. 1: however, in exp. 2 PEC were unresponsive to a broad range of treatment doses. Neither was IFN detected in culture supernatants of CL-246,738 treated human lymphocytes. When supernatants were harvested 48 hrs versus 24 hrs after CL-246,738 induction, no difference in the results was observed. The highest dose of CL-246,738 that could be used in tissue culture, without being toxic, was 10 µg/ml. Ampligen, used as a positive control (50 µg/ml) always induced 81-243 units of IFN in these cell cultures. Thus, CL-246,738 cannot be used in tissue culture systems as an IFN inducer. The drug may need to be metabolized in vivo in order to become an IFN inducer. The usefulness of the drug in human systems cannot be established by this approach (Table 17).

Discussion and Conclusions

Most of the preliminary work needed to design meaningful trials of combination therapies against model arboviruses has been accomplished during the initial year of the project, and combination experiments in vivo have begun. Included in the preliminary work has been growing of stock viruses, preparation of immune globulins, establishment of tissue culture assay systems for each virus, and establishment of animal model systems.

Broad scale tissue culture screens of candidate antivirals have allowed us to make initial priority decisions for in vivo experiments. Based on the data presented in Tables 2-6, CH_3NH_2HCl, theophylline, phenytoin, Mo-CVI and amantidine have been placed on hold as candidates for in vivo studies, while the IFNs, chloroquine, ribavirin, CL-246,738 and the non-peptide CVIs remain high on our list of candidate compounds. Other candidate compounds include the various immune modulators, which could not be evaluated in these tissue culture screens, and immune globulins prepared by us.

A number of tentative conclusions can be drawn from the interferon titrations (Table 2). First, both mouse IFNα/β and human rIFNα A/D seem to show high activity against Semliki Forest, Banzi, West Nile, Bunyamwera and LaCrosse virus, with markedly lower activity against Sindbis virus. Second, the highly species specific action of mouse IFNγ makes comparative evaluation difficult, but both SF and WN viruses seem quite sensitive to its action. Third, the crude human IFNγ preparation seems to be less active against Bunyamwera than SF or LAC viruses. All of the results from IFN titrations will need to be confirmed. It will be particularly important to test different IFN/virus combinations on the same cell lines in order to more accurately identify the cause of the variation seen. Finally, comparison of the in vitro and in vivo results for murine IFNα/β against SF (Tables 2,9) and BZ (Tables 2,12) again illustrate how vital it is to confirm tissue culture results in animal models.

Single drug dose response experiments have allowed us to determine initial priorities for combination therapy experiments. Initial focus in combinatorial studies will be poly I:CLC, murine IFNα/β, neutralizing antibodies, and CL-246,738. Other compounds which will probably be added to the list, based on the results of ongoing dose response experiments, are ribavirin, IFNγ, non-peptide CVI, MD(T)P, TNF and C. parvum. Results from the first combination experiment, using BZ antigen and poly I:CLC (Table 16), indicate that our protocol will be effective in determining interactions of drugs in vivo.

Two similar immunomodulating drugs, Ampligen and poly I:CLC, were compared to test their effectiveness as antiviral agents. As shown in Table 14, Ampligen, at a dose of 8×10^{-1} mg/kg, fully protected weanling mice against a lethal challenge with Banzi virus when given intraperitoneally. Ampligen was also totally protective against Semliki Forest virus at doses as low as 8×10^{-3} mg/kg (Table 8). By contrast, poly I:CLC gave full protection against Banzi virus at a dose as low as 4×10^{-2} mg/kg (Table 14). Thus, poly I:CLC was 20-fold more effective than Ampligen.

We had shown Banzi to be very sensitive to the action of IFN in vitro (Table 2). Thus, we investigated whether the greater efficacy of poly I:CLC versus Ampligen against Banzi virus correlated with a greater ability to induce IFN in vivo. As shown in Table 18, the IFN titers obtained in the serum and in the peritoneal cavity of mice injected with poly I:CLC 6 hrs earlier was much greater, on a per dose basis, than the IFN titers of mice injected with Ampligen. The IFN titers peaked at 6 hrs and declined 24 hrs after drug administration (data not shown). In summary, poly I:CLC seems to be a more effective antiviral drug than Ampligen, and as such it will be employed in future experiments.

We also evaluated the protection produced by the immunomodulator, CL-246,738. This drug, given orally, protected mice against lethal challenge with SFV or Banzi virus (Tables 10,15). In these experiments, a single oral dose of CL-246,738 was given 24 hrs before injection with the virus: full protection against the two viruses was obtained with drug's doses ranging from 0.1 mg/mouse (5 mg/kg) to 3 mg/mouse (150 mg/kg). High doses of the compound were potent inducers of interferon in serum and peritoneal fluid (Fig. 4). In fact, the levels detected in the peritoneal fluid are an underestimate, since 2 ml of media was added to the peritoneal cavity as a lavage to collect the induced IFN. Thus, the amounts of IFN detected (26-358 units/ml) represents a 10- to 100-fold dilution of the concentrations actually present in the peritoneal cavity.

Interferon was produced by both spleen and peritoneal exudate cells harvested from mice pretreated with CL-246,738. Interestingly, the kinetics of this induction were not the same in both cell types (Fig. 5). However, no consistent IFN production was seen when cells from untreated mice were induced in vitro with CL-246,738 (Table 17). This may indicate that a metabolic product of the compound is the actual inducer of IFN. Unfortunately, this finding makes it impossible to easily determine whether CL-246,738 is an effective IFN inducer in man (Table 17). It is not, however, certain that the only, or even the major, protective effect of CL-246,738 in vivo comes from the induction of IFN. For example, this compound produced complete protection in the BZ model system when a single oral dose (0.1 mg) was given prior to virus challenge (Table 15). This dose of CL-246,738 produced a 24 hr serum IFN titer of 200 U/ml (Fig. 4), which decreased to undetectable levels by 48 hrs. However, when IFNα/β was used

in the same system, no protection was seen even when high doses (10,000 units/mouse) were administered (Table 12). Thus, this IFN dose, given i.p., was either not sufficient to perceptibly raise baseline IFN levels *in vitro*, which would be inconsistent with the parallel experiment in the SF model system (Table 9), or the major *in vivo* protective effects of CL-246,738 are through other immunomodulatory pathways.

TABLE 1. Tissue Culture Systems for Virus Assays

Virus	Cells		Methyl Cellulose Overlay	Incubation Time
	Preferred	Alternate		
Alphaviruses				
Semliki Forest	VERO	CER, L929	0.5%	28 hr.
Sindbis	CER	WISH	0.8%/0.5%	25 hr.
Flaviviruses				
Banzi	CER	VERO	0.8%	48 hr.
West Nile	L929	CER, WISH	0.5%	48 hr.
Bunyaviruses				
Bunyamwera	CER	VERO	0.5%	45 hr.
La Crosse	VERO	CER	0.5%	55 hr.
Arenavirus				
Pichinde	MA-104	-	0.5%	6 days

TABLE 2. Effect of Interferon Treatment on Arbovirus Replication

Virus (cell)	Interferon			
	Mu α/β (100,000 U/ml) Protective Titer	Mu γ (1,000 U/ml) Protective Titer	Hu rαA/D (1,000 U/ml) Protective Titer	Crude Hu γ (1,000 U/ml) Protective Titer
Alphavirus				
Semliki Forest (VERO)	< 3	< 3	243	162
(L929)	>59,049	1,458	19,683	< 3
Sindbis (CER)	6,561	< 3	1,458	3
Flavivirus				
Banzi (CER)	59,049	3	4,374	3
West Nile (L929)	>59,049	1,458	4,374	< 3
Bunyavirus				
Bunyamwera (CER)	39,366	< 3	2,187	6
(VERO)	< 3	< 3	729	9
LaCrosse (CER)	59,043	< 3	2,187	< 3
(VERO)	< 3	< 3	2,187	729

TABLE 3. Effect of Antiviral Compounds on Arbovirus Replication in Tissue Culture With Drug Removed After 2 hr Incubation

Compound	Minimum Inhibitory Concentration[1]					
	SB[2]	SF[3]	BZ[4]	WN[5]	BW[6]	LAC[7]
NH_4Cl	0.195	0.260	1.040	0.521	0.521	0.200
$NH_4C_2H_3O_2$	0.260	0.391	1.560	0.781	0.781	0.200
NH_4NO_3	0.195	0.391	1.040	0.521	0.521	0.200
CH_3NH_2HCl	>0.500	0.500	>0.500	>0.500	>0.500	0.140
Chloroquine	0.008	0.100	0.010	0.005	0.004	0.008
CL-246,738	0.002	0.002	0.004	0.002	≥0.006	0.002
Amantidine	0.030	0.500	>0.500	0.125	>0.500	≥0.250
Rimantidine	0.170	0.125	0.250	0.042	0.250	0.083
Ribavirin	0.063	0.500	0.170	0.040	0.004	0.010
Theophylline	>0.500	>0.050	>0.500	>0.500	>0.500	>0.500
Phenytoin	>0.250	>0.500	>0.250	>0.250	>0.250	>0.250

[1] MIC for 50% plaque reduction, in mg/ml
[2] Sindbis virus on WISH cells
[3] Semliki Forest virus on VERO cells
[4] Banzi virus on CER cells
[5] West Nile virus on CER cells
[6] Bunyamwera virus on CER cells
[7] La Crosse virus on CER cells

TABLE 4. Effect of CVI Preparations on Arbovirus Replication in Tissue Culture With Drug Removed After 2 hr Incubation

	Antiviral Titer [1]					
Preparation	SB [2]	SF [3]	BZ [4]	WN [5]	BW [6]	LAC [7]
P-8407	24	64	16	32	32	32
PX-861	24	64	<2	16	48	48
PX-862	<2	48	<2	<2	<2	<2
PX-863	48	64	<2	48	64	48
MO-CVI	<2	<2	<2	<2	<2	<2
Milk Extract	>256	48	8	32	>256	>256

[1] 50% plaque reduction titer
[2] Sindbis virus on WISH cells
[3] Semliki Forest virus on VERO cells
[4] Banzi virus on CER cells
[5] West Nile virus on CER cells
[6] Bunyamwera virus on CER cells
[7] La Crosse virus on CER cells

Table 5. Effect of Antiviral Compounds on Arbovirus Replication in Tissue Culture Using a Continuous Overlay System

Compound	Minimum Inhibitory Concentration[1]					
	SB[2]	SF[3]	BZ[5]	WN[5]	BW[6]	LAC[7]
NH_4Cl	0.260	0.521	0.521	0.521	0.521	0.521
$NH_4C_2H_3O_2$	0.390	0.781	0.781	0.521	1.040	0.521
NH_4NO_3	0.260	0.781	0.521	0.521	0.781	0.391
CH_3NH_2HCl	$\geq$0.500	0.167	$\geq$0.500	>0.500	>0.500	0.500
Chloroquine	0.010	0.010	0.021	0.010	0.042	0.063
CL-246,738	>0.013	0.004	0.004	0.003	0.006	0.008
Amantidine	0.250	>0.042	>0.500	0.250	>0.250	0.083
Rimantidine	>0.125	>0.063	0.125	0.042	>0.125	0.167
Ribavirin	N.D.	0.167	>0.125	N.D.	>0.125	0.042
Theophylline	>0.500	>0.250	>0.500	>0.500	>0.500	>0.500
Phenytoin	>0.050	>0.025	>0.050	>0.050	>0.050	>0.050

1. MIC for 50% plaque reduction, in mg/ml
2. Sindbis virus on WISH cells
3. Semliki Forest virus on VERO cells
4. Banzi virus on CER cell
5. West Nile virus on CER cells
6. Bunyamwera virus on CER cells
7. LaCrosse virus on VERO cells

Table 6. Effect of CVI Preparations on Arbovirus Replication in Tissue Culture Using a Continuous Overlay System

CVI Preparation	Antiviral Titer[1]					
	SB[2]	SF[3]	BZ[4]	WN[5]	BW[6]	LAC[7]
P-8407	32	48	24	<256	64	32
PX-861	16	4	<8	24	48	32
PX-862	16	48	<32	<256	48	128
PX-863	32	24	<32	<128	64	96
MoCVI	<2	6	<2	2	<2	6
Milk Extract	>256	6	<4	32	>256	128

1. 50% plaque reduction titer
2. Sindbis virus on WISH cells
3. Semliki Forest virus on VERO cells
4. Banzi virus on CER cell
5. West Nile virus on CER cells
6. Bunyamwera virus on CER cells
7. LaCrosse virus on VERO cells

Table 7. Summary of Antibody Production

Virus	Ascitic Fluid		CDR Purified Antisera[1]	
	Volume (ml)	Avg. Titer	Volume	Titer
Alphaviruses				
Semliki Forest[2]	116	48	135	16
	116	16	105	≤4
	52	128		
Sindbis[3]	197	32	N.D.	N.D.
Flaviviruses				
Banzi[3]	188	256	115	3,840
	50	≥2,048	77	5,120
West Nile[4]	89	≥30,000	N.D.	N.D.
Bunyaviruses				
Bunyamwera[3]	86	≥4,096	150	3,840
	177	≥4,096	130	900
La Crosse[3]	26	≥8,192	N.D.	N.D.

[1]Clarified with Whatman CDR Cellulose, and filter sterilized.
[2]Assayed on VERO cells.
[3]Assayed on CER cells.
[4]Assayed on L929 cells.

Table 8. In Vivo Protection Against Semliki Forest Virus With Ampligen (PolyI:C)

Drug Dose mg/kg B.W.	Virus Dilution	Virus Dose LD_{75}	# Mice	% Mortality	% Protection Relative to Control
8.0	$10^{-6.0}$	3	5	0	100
0.8	$10^{-6.0}$	3	5	0	100
0.08	$10^{-6.0}$	3	5	0	100
0.008	$10^{-6.0}$	3	5	0	100
0.0008	$10^{-6.0}$	3	4	25	75
None	$10^{-6.0}$	3	4	100	N.A.

The drug (0.2 ml) was injected i.p. at -6 hrs

Table 9. In Vivo Protection Against Semliki Forest Virus With Murine IFN α/β

Drug Dose Units/Mouse	Virus Dilution	Virus Dose LD_{75}	# Mice	% Mortality	% Protection Relative to Control
10,000	$10^{-6.0}$	3	5	40	60
10,000	$10^{-6.5}$	1	5	0	100
300	$10^{-6.0}$	3	5	80	20
300	$10^{-6.5}$	1	5	40	50
10	$10^{-6.0}$	3	5	100	0
10	$10^{-6.5}$	1	5	60	25
None	$10^{-6.0}$	3	5	100	NA
None	$10^{-6.5}$	1	5	80	NA

The drug (0.2 ml) was injected i.p. at -1 hr

Table 10. In Vivo Protection Against Semliki Forest Virus With CL-246,738

Drug Dose (mg/Mouse)	Virus Dilution	Virus Dose LD_{75}	# Mice	% Mortality	% Protection Relative to Control
3.0	$10^{-6.0}$	3	5	0	100
1.0	$10^{-6.0}$	3	5	0	100
0.3	$10^{-6.0}$	3	5	0	100
0.1	$10^{-6.0}$	3	5	0	100
None	$10^{-6.0}$	3	5	100	N.A.

The drug (0.1 ml) was administered orally at -24 hrs

Table 11. In Vivo Protection Against Banzi Virus with Chloroquine Administered Prophylactically at Time -24 and 0 hr

Drug Concentration[1]	Virus Dilution	Virus Dose LD_{75}	# Mice	% Mortality	% Protection Relative to Control
15 mM	$10^{-6.0}$	3	10	50	37
15 mM	$10^{-6.5}$	1	10	30	62
15 mM	-	-	5	0	N.A.
10 mM	$10^{-6.0}$	3	10	80	0
10 mM	$10^{-6.5}$	1	10	50	0
10 mM	-	-	5	0	N.A.
-	$10^{-6.0}$	3	10	80	N.A.
-	$10^{-6.5}$	1	10	50	N.A.

[1] 0.2 ml of chloroquine solution at the indicated concentrations were administered i.p. at the stated times.

Table 12. Lack of In Vivo Protection Against Banzi Virus With Murine IFN α/β

Drug Dose Units/Mouse	Virus Dilution	Virus Dose LD_{75}	# Mice	% Mortality	% Protection Relative to Control
10,000	$10^{-6.0}$	3	5	100	0
10,000	$10^{-6.5}$	1	5	100	0
300	$10^{-6.0}$	3	5	100	0
300	$10^{-6.5}$	1	5	100	0
10	$10^{-6.0}$	3	5	100	0
10	$10^{-6.5}$	1	5	100	0
None	$10^{-6.0}$	3	5	100	NA
None	$10^{-6.5}$	1	5	100	NA

The drug (0.2 ml) was injected i.p. at -1 hr

Table 13. In Vivo Protection Against Banzi Viruses With Polyclonal Antisera[1]

Time of Treatment (hr)	Virus Dilution	Virus Dose LD_{75}	# Mice	% Mortality	% Protection Relative to Control
-24	$10^{-6.0}$	3	5	0	100
	$10^{-6.5}$	1	5	0	100
+2	$10^{-6.0}$	3	5	0	100
	$10^{-6.5}$	1	5	0	100
+24	$10^{-6.0}$	3	5	40	60
	$10^{-6.5}$	1	5	20	73
+48	$10^{-6.0}$	3	5	100	0
	$10^{-6.5}$	1	5	40	46
+72	$10^{-6.0}$	3	5	100	0
	$10^{-6.5}$	1	5	20	73
Control	$10^{-6.0}$	3	5	100	N.A.
	$10^{-6.5}$	1	5	80	N.A.

[1]250 Neutralizing units of antibody were administered i.p. at the stated times relative to administration of virus challenge.

Table 14. Comparison Between Ampligen and PolyI:CLC for Protection In Vivo Against Banzi Virus

Ampligen			PolyI:CLC		
Drug Dose (mg/kg)[a]	% Mortality	% Protection	Drug Dose (mg/kg)[a]	% Mortality	% Protection
8	0	100	4	0	100
0.8	0	100	0.4	0	100
0.08	20	80	0.04	0	100
0.008	20	80	0.004	40	60
0.0008	100	0	ND	--	--
None	100	NA	None	100	NA

[a]0.5 ml of drug at the indicated doses was given i.p. at -6 hrs (5 mice/group); $3LD_{75}$ of Banzi virus was given i.p. at 0h.

Table 15. In Vivo Protection Against Banzi Virus With CL-246,738

Drug Dose (mg/Mouse)	Virus Dilution	Virus Dose LD_{75}	# Mice	% Mortality	% Protection Relative to Control
3.0	$10^{-6.0}$	3	5	0	100
1.0	$10^{-6.0}$	3	5	0	100
0.3	$10^{-6.0}$	3	5	0	100
0.1	$10^{-6.0}$	3	5	0	100
None	$10^{-6.0}$	3	5	100	N.A.

The drug (0.1 ml) was administered orally at -24 hrs

Table 16. Combination Therapy Against Banzi Virus With Immune Globulin and PolyI:CLC

Drug Dose		Nominal Virus Dose		% Protection Relative to
Antibody (Units)	PolyI:CLC (mg/kg Body Weight)	LD_{75}	% Mortality	Control
150	4×10^{-3}	3	0	100
150	2×10^{-3}	3	0	100
150	4×10^{-4}	3	0	100
150	0	3	0	100
60	4×10^{-3}	3	0	100
60	2×10^{-3}	3	0	100
60	4×10^{-4}	3	0	100
60		3	20	75
25	4×10^{-3}	3	0	100
25	2×10^{-3}	3	20	75
25	4×10^{-4}	3	0	100
25		3	60	25
0	4×10^{-3}	3	0	100
0	2×10^{-3}	3	20	75
0	4×10^{-4}	3	40	50
0	0	3	80	NA

Antibody was administered i.p. at -24 hrs, PolyI:CLC administered i.p. at -6 hrs, relative to virus inoculation. 10^{-6} dilution of stock virus administered i.p. (5 mice/group) to all mice except for drug (toxicity) controls.

Table 17. Lack of IFN Production by Cells Treated In Vitro with CL-246,738

Exp.	Dose of Drug (µg/ml)	IFN Titer: Murine Cells[a] Spleen	PEC	PBL	Human Cells[a] Spleen	PBL
1	5	<3	81	ND	<3	<3
	2.5	<3	54	ND	9	<3
	-	<3	27	ND	9	<3
2	10	<3	<3	<3	<3	ND
	5	<3	9	<3	<3	<3
	2.5	<3	<3	<3	<3	<3
	1.2	<3	<3	<3	<3	ND
	0.6	<3	3	<3	3	ND
	0.3	<3	9	<3	3	ND
	0.1	<3	18	<3	3	ND
	0.08	<3	<3	<3	3	ND
	-	<3	<3	<3	3	<3

[a]Cells were plated at 4 x 10^5/well of a 96-well plate and treated with CL-246,738 at the doses indicated for 24 hrs. Culture supernatants were then tested for IFN activity in a standard IFN assay.

Table 18. Comparison Between Ampligen and PolyI:CLC for IFN Induction In Vivo

Ampligen Induction			PolyI:CLC Induction		
Drug Dose (mg/kg)[a]	Serum[b]	i.p.[c]	Drug Dose (mg/kg)[a]	Serum[b]	i.p.[c]
8	3,013	68	4	>6561	ND
0.8	83	ND	0.4	46260	1791
0.08	<30	5	0.04	2283	ND
0.008	<30	ND	0.004	140	ND
0.0008	<30	<3	ND	--	--
None	<30	<3	None	<30	<3

[a] see Table #14
[b] average IFN titer (U/ml) of serum samples taken at 0h from 3 mice/group
[c] average IFN titer of peritoneal lavages done at 0h to 3 mice/group (2 ml/mouse)
ND - not determined

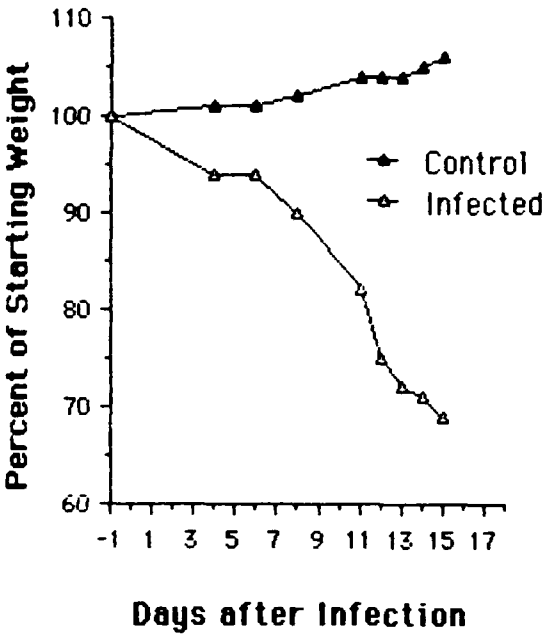

Figure 1. Effect of Pichinde Infection on Strain 13 Guinea Pigs

Figure 1: Effect of Pichinde Infection on Strain 13 Guinea Pigs. Average weight loss of Strain 13 guinea pigs challenged with passage 14 Pichinde virus, compared to uninfected controls. Weights are expressed as a percent of the average starting weight.

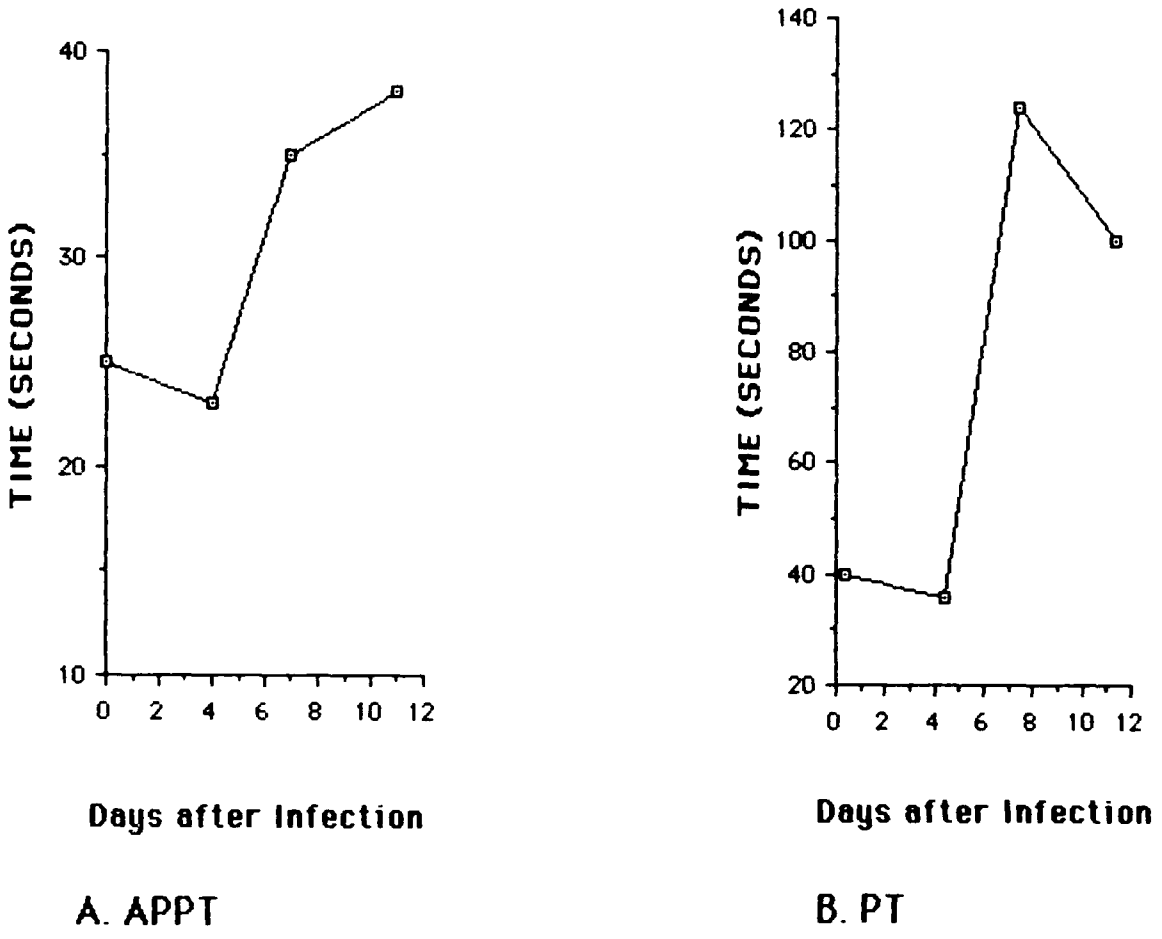

Figure 2: Change in Clotting Times in Strain 13 Guinea Pigs After Infection with Pichinde Virus.

Plasma from infected animals was collected at days 4, 7, and 11 after infection. Clotting times for partial thromboplastin (APPT, Panel A) and prothrombin (PT, Panel B) assays are shown. Day 0 values are averages for uninfected guinea pigs: APTT normal range = 21-28 secs; PT normal range = 30-40 secs.

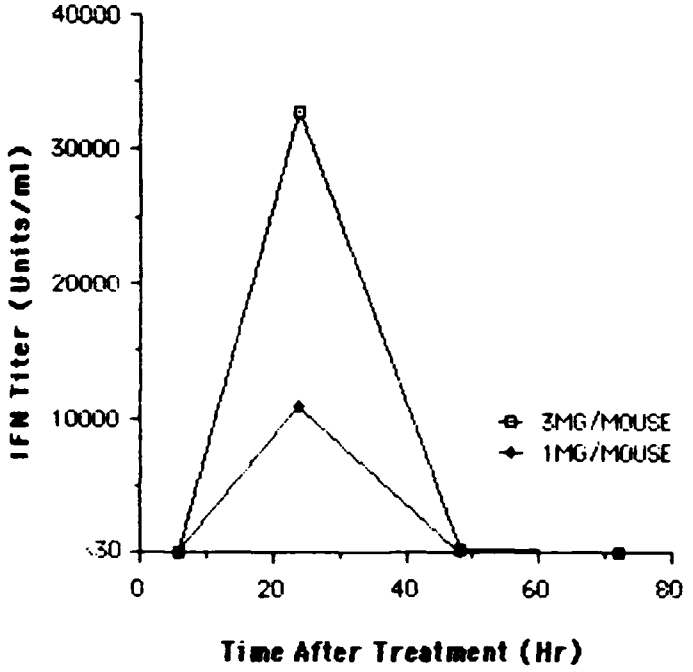

FIGURE 3. KINETICS OF SERUM IFN PRODUCTION IN CL-246,738 INDUCED MICE

Figure 3: Kinetics of Serum Interferon Production in CL-246,738-Induced Mice.

Weanling mice were treated orally with CL-246,738 at 0, +24, and +48 hrs. Treated mice were bled at 6, 24, 48, and 72 hrs, and titers of circulating interferon determined.

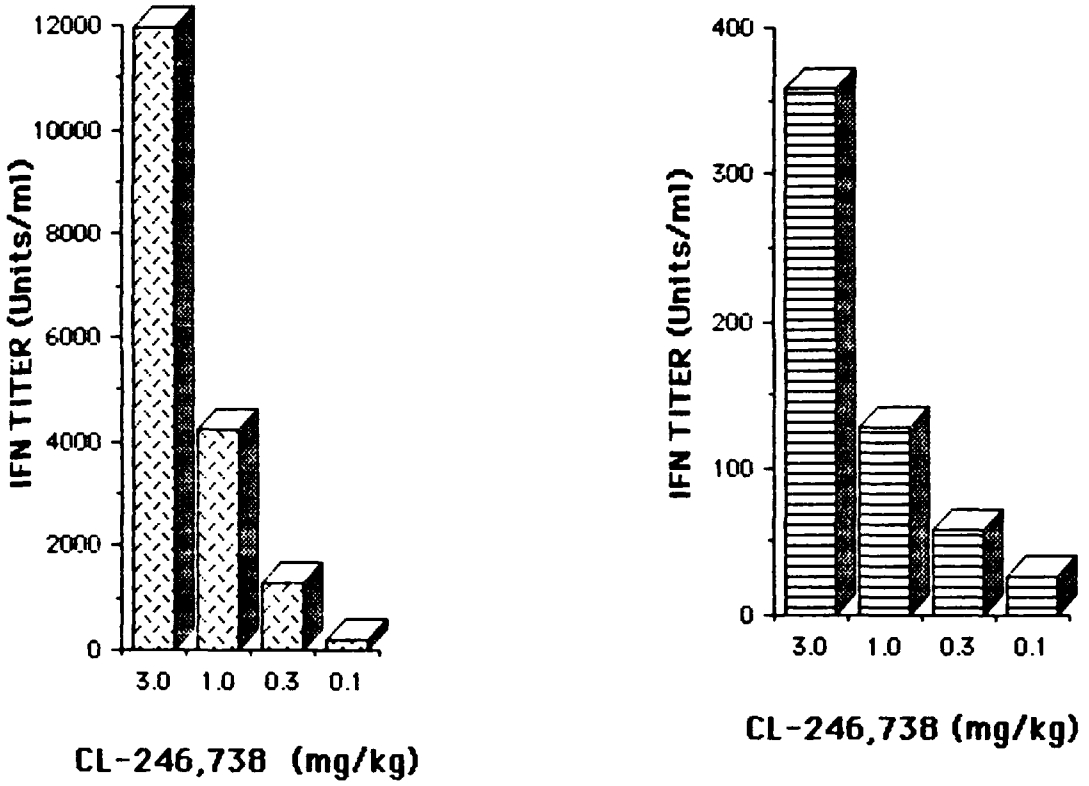

Figure 4: Dose Response of Interferon Production in the Serum and Peritoneal Cavity of CL-246,738 Treated Mice.

Mice were treated orally with the indicated doses of CL-246,738. Twenty-four hrs later blood was drawn from half the mice. The remaining mice were sacrificed. 2 ml of EMEM was injected into the peritoneal cavities as a lavage. The media was immediately withdrawn, and interferon titers were determined.

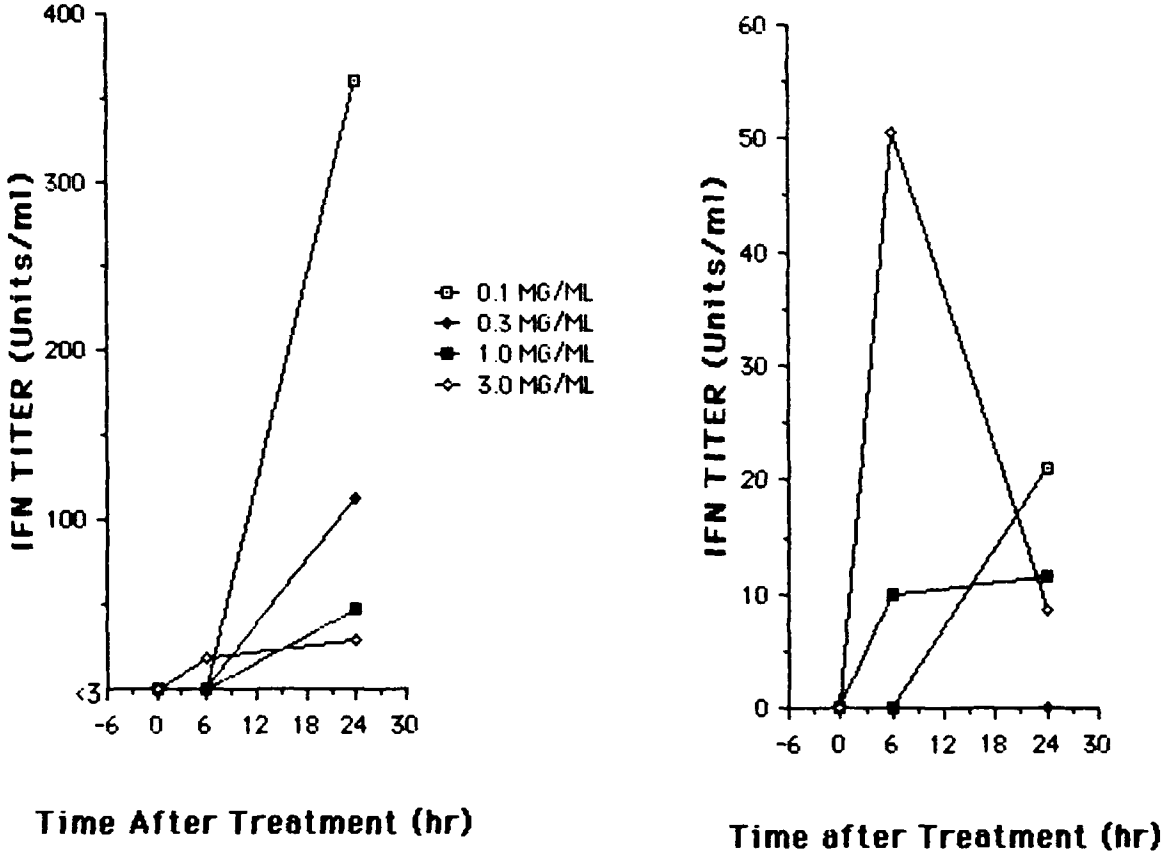

Figure 5: Induction of Interferon in Peritoneal Exudate Cells and Spleen Cells from CL-246,738 Treated Mice.

Peritoneal exudate cells (Panel A) or spleen cells (Panel B) were prepared from mice pretreated with CL-246,738. Cells were cultured in vitro for 24 hrs, after which the supernatants were tested for IFN activity.

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